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Photorhabdus luminescens is an insect pathogenic bacterium that is symbiotic with entomopathogenic nematodes. Upon invasion of insect larvae, P. luminescens is released from the nematodes and kills the insect mainly through the action of large tripartite ABC-type toxin complexes (Tcs). Tcs are typically composed of TcA-, TcB- and TcC proteins that are only biologically active when complete. Functioning as ADP-ribosyltransferases, TcC proteins were identified as the actual functional components that induce actin-clustering, defects in phagocytosis and cell death. However, little is known about the translocation of TcC into the cell by the TcA and TcB components. Here, we show that TcA (TcdA1) forms a transmembrane pore and report its structure in the prepore and pore state determined by cryo-electron microscopy. We found that the TcdA1 prepore assembles as a pentamer forming a α-helical vuvuzela-shaped channel less than 1.5 nm in diameter surrounded by a large outer shell. Membrane insertion is triggered not only at low pH as expected, but also at high pH values, suggesting a novel route for Tc toxin action directly through the midgut of insects. Comparisons with structures of the TcdA1 pore inserted into a membrane and in complex with TcdB2 and TccC3 reveal large conformational changes during membrane insertion suggesting a novel syringe-like mechanism of protein translocation. Our results demonstrate how ABC-type toxin complexes bridge a membrane to insert their deadly components into the cytoplasm of the host cell. Our proposed mechanism is paradigmatic for the whole ABC-type toxin family. It is an important step towards the understanding of the host-pathogen interaction and the complex life cycle of Photorhabdus luminescens and other pathogens, including human pathogenic bacteria, and serves as a strong foundation for the development of biopesticides.

1809-Pos Board B701
Conformational Transitions Driving Lattice Growth at an S-Layer Boundary Resolved by cryo-TEM
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S-layers are a two-dimensional protein or glycoprotein lattices that cover the surfaces of many bacteria and archaea. Their nanoscale periodic, porous structure and relative ease of manipulation give them great potential for nanobiotechnological applications. However, details of the assembly process are not yet known for any S-layer and high resolution structural information is still very limited. We report a two-dimensional (2D) structural analysis of the expanding boundary of isolated Lysinibacillus sphaericus S-layer (SbpA) growing on a graphene support. The unique physical properties of graphene offer great potential for superior image quality in cryogenic transmission electron microscopy (cryo-TEM) of biological macromolecules; however, as yet no direct application has been reported. In vitro reconstitution of disassembled S-layer monomers leads to free-standing 2D lattices with long-range order in the presence of Ca^{2+} ions. To gain insight into the assembly in the intact solution state, we obtained cryo-TEM images of single sheets plunge-frozen while growing on graphene. As the active self-assembling S-layers are instantly frozen all conformational states present at the expanding boundary on the graphene flat support are captured. Selected image sections provide a view of the steps leading to subunit recruitment and maturation in S-layers self assembly. We find that the SbpA homotetrameric subunits spanning the lattice are not preassembled and fully folded prior to incorporation. Instead, the addition of monomers to the open boundary happens in concert with the maturation of the adjacent homotetrameric subunits. This work shows the use of graphene support to enable the solution of a 2D problem while optimizing high quality cryo-TEM data. These results open the way for higher resolution, time-resolved structural analysis of the critical domains and interactions in wild-type and engineered S-layers.

1810-Pos Board B702
Cryo-Electron Tomography of Mitochondria in Intact Cells Reveals Mitochondria Divide by Budding
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Eukaryotes rely on mitochondrial division so that new generation of cells can acquire adequate number of mitochondria to maintain their physiological functions. Mitochondrial division has long been thought to occur by binary fission which has recently been considered to be mediated by Drp1 and ER. However, the ultimate verification of the process of mitochondrial division has previously depended heavily on the visualization by fluorescent microscopy and conventional two-dimensional (2D) electron microscopy (EM). The resolution limit of fluorescent microscopy essentially prevents observers from seeing sufficient details of mitochondrial division which involves distance between two separating mitochondrial bodies down to zero. In conventional EM, specimen usually goes through extensive chemical and mechanical treatments including fixation, dehydration, staining and sectioning which may readily disrupt critical structure features that define mitochondrial division. On the other hand, in conventional EM, fine structure features always tend to be buried in 2D images due to overlapping of three-dimensional information. Here, I utilized the Whole Cell Cryo-Electron Tomography to probe mitochondrial division in intact cells. Small mitochondria were clearly observed budding from large mitochondria. Snapshots of large number of intermediates of mitochondrial budding were captured showing relatively larger mitochondrial buds of various sizes connected to large mitochondria by weak tethers or stalk-like structures essentially forming mitochondrial networks. High contrast densities most likely corresponding to mitochondrial DNA could be seen inside each of the mitochondrial buds. Moreover, mitochondrial budding resembles the reproductive budding of alpha-proteobacteria from which mitochondria are believed to be evolved. Therefore, this study has revealed that mitochondria divide by budding.

1811-Pos Board B703
Three-Dimensional Visualization of Whole Synapses by Stem Tomography
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Scanning transmission electron microscope (STEM) tomography enables determination of 3D ultrastructure from 1 or 2-micrometer thick sections of cells and tissues. These sections are considerably thicker than can be analyzed by conventional electron tomography, where resolution is limited by chromatic aberration due to multiple inelastic scattering. In STEM tomography a probe of small angular convergence gives a large depth of field throughout the thickness of the specimen while maintaining a probe diameter of approximately 2 nm; and the use of an on-axis bright-field detector reduces the effects of beam broadening and thus improves the spatial resolution compared to that attainable by STEM dark-field tomography. We have found that STEM tomography is ideal for visualizing entire synapses in the nervous system, and for making quantitative measurements on the numbers, sizes and shapes of synaptic components. We applied the technique to study the architecture of ribbon synapses in retina, and the structure of postsynaptic densities in brain cortex. For the first time, it was possible to determine a full 3D architecture of ribbon synapses in mammalian (rat) rod bipolar cells, in which regular docked and tethered vesicles, as well as larger (possibly pre-fused) vesicles were visualized. Quantitative analysis revealed a readily releasable pool of vesicles, which correlates structurally with previous physiological data. We have also applied STEM tomography to reconstruct entire spine postsynaptic densities in mouse hippocampus, both in control preparations as well as in preparations, where RNAi knockdown eliminates specific PSD scaffolding proteins to illustrate their key role in organizing the PSD. STEM tomography of thick sections thus provides a novel approach for correlating the nanoscale structure of synapses with function.
This work was supported by the Intramural Research Programs of NIBIB and NINDS, NIH.

1812-Pos Board B704
Opening Windows into the Cell: Focused Ion Beam Micromachining of Eukaryotic Cells for Cryo-Electron Tomography
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Cryo-electron tomography (cryo-ET) provides unprecedented insights into the 3-D macromolecular organization of cells in their native state. However, the thickness of most cells makes them inaccessible to cryo-ET. Focused ion beam (FIB) milling can be used to prepare 200-500 nm lamellae from intact cells, opening large windows into the cell's interior, exposing their landscapes at molecular resolution. Advanced computational analysis makes the identification of macromolecular complexes possible, yielding visual proteomes of cells. Cryo-ET and FIB milling were used to study the structural dynamics of the nuclear pore complex, one of the largest macromolecular machines in the cell. It is

composed of hundreds of proteins, selectively controlling all traffic between the nucleus and the cytoplasm. The architecture of the NPC is central to understanding nuclear transport. However, due to its sheer size, its local environment and its dynamic nature, determining its structure at molecular resolution remains a challenge for conventional techniques. Combining FIB milling, cryo-ET, and image processing enables the study of the NPC in its native environment, free of the distortions caused by purification. This approach has not only revealed the NPC architecture at unparalleled resolution, but also captured different conformational states in action. Other uses of cryo-FIB/ET to study diverse cellular environments at molecular detail will be presented, including actin networks, the architecture of cell division, and the distribution of macromolecular complexes within organelles such as mitochondria.

1813-Pos Board B705

Development for Dynamic Live Cell Imaging by Cryo-Electron Tomography and Stem

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Cryo-EM tomography of intact cells is an emerging technology that compliments crystallography, NMR and single molecule imaging techniques. Its strength is in that it reveals the spatial arrangements of key proteins and complexes during intracellular signaling and mechanical events like motility and division. In this meeting, we describe dynamical Live Cell imaging by Cryo-EM tomography using the Titan Krios microscope with 300 kV electron column and HAADEF detector. Observed living cells were grown directly on Holey Carbon Support Film. In order to make sure that the cells are suitable for observation we analyzed the some parameters of cell movement and cell division on Quantifoil using Fiji and compared these properties with those of cells grown on a normal cell culture plastic plate. After phosphate-buffered saline washing, the cells were done rapid freeze fixation (vitrification) by dropping in liquid ethane using Vitrobot^{TM} Mark IV instead of the usual chemical fixation and were transferred onto the microscope immediately while keeping the environment under liquid N_2. Cells were imaged over an angular range from −70 degrees to 70 degrees at 2 degrees x cos θ tilt increments automatically and analyzed with Inspect 3D and Amira software to provide 3D images and Volume rendering respectively.

We observed some unique architectures of a part of Lamellipodium in GFP-Myosin X expressed COS 7 cell and them from near the nuclear membrane to plasma membrane in COS 7 and several eukaryotic cells (HeLa, NIH3T3⋯ etc.) by Cryo-EM tomography using STEM using intact cells and vitreous cell sections.

At future, our system can provide any new information about many kinds of cells and organelles during important events.

1814-Pos Board B706

New Method for Applying Multiple Samples to a TEM Specimen Grid

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Transmission electron microscopy (TEM) enables visualization and structural determination of biological macromolecules. Examination of several samples by TEM is greatly limited by the time that it takes to insert each sample on the microscope, and the deteriorating vacuum on the TEM after this process is repeated several times. Nevertheless, when observing a specimen by TEM, in almost all cases the examined area of the grid represents a small fraction of the available area. Thus, in theory, a TEM grid has space for more than one sample. Here we have exploited the microarray technology to apply multiple samples to a single TEM specimen grid. Microarrayers can deposit small quantities of sample using a printer-like machine that transfers the liquid from the reservoir to the assay surface. We demonstrate that microarray technology can be used to accurately position picoliter quantities of sample within micrometric distances in the reduced space of the TEM grid with negligible cross-contamination. This technique permitted the deposition of samples into arrays of 2 to 36 discrete spots on a single TEM grid. The TEM grid containing a sample microarray can be negatively stained. After introducing the microarray grid in the microscope every spot can be inspected and images can be recorded digitally or on film. In conclusion, the microarray method can dramatically decrease the time necessary for TEM grid preparation and examination at least by one order of magnitude, while preserving the vacuum of the electron microscope. This new method is very suitable for screening and data collection in experiments that generate a multiplicity of samples.

1815-Pos Board B707

Direct Visualization of HIV-1 with Correlative Live-Cell Microscopy and Cryo-ElectronTomography

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Cryo-electron tomography (cryoET) allows 3D visualization of cellular structures at molecular resolution in a close-to-physiological state. However, due to the poor signal, low contrast, and radiation sensitive nature of unstained frozen-hydrated specimens, acquisition of tomographic projection series is not generally selective in choice of imaging targets. Therefore, the full potential of cryoET for 3D cellular imaging is realized, especially for cellular processes that are rare or dynamic. In order to overcome this limitation in cryoET analysis, approaches for correlating fluorescent light microscopy and cryoET are highly desirable, not only to complement the structural information obtained from cryoET with the dynamic functional data from fluorescent labeling, but also to guide sampling in cryoET. Such tools are particularly valuable for investigating the early events of HIV-1 infection in cells, which are infrequent and difficult to catch. Here, we report on a methodology that combines high speed 3D live-cell imaging with cryoET tools. We applied this technology to visualize the process viral entry into HeLa cells, following of the same particles. Through direct 3D visualization we identified HIV-1 particles that are smaller than the diffraction limit of light microscopy (~ 100 nm). They were found attached to plasma membrane and in MVBs after cell entry. We also showed, for the first time under near-native conditions, that intact hyperstable mutant HIV-1 cores are released into the cytoplasm of host-cells. We anticipate that the methodology established here will not only constitute a useful tool for studying virus-host cell interactions at various stages during infection, but will also open new ways to investigate cell signaling events and many other cellular processes in general.

1816-Pos Board B708

Structural Plasticity within the Postsynaptic Density

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The postsynaptic density (PSD) is a large protein complex that clusters neurotransmitter receptors at the synapse and organizes the intracellular signaling molecules responsible for altering the efficiency of synaptic transmission – termed synaptic plasticity. We propose that synapses from different parts of the brain place unique demands on the process of synaptic transmission and that the structure and composition of the PSD play a role in providing these distinctive properties. To begin to address this question, PSDs were isolated from adult rat cerebella, hippocampi and cortices, three brain areas amenable to straightforward isolation that contain unique distributions of neuronal cell types. Electron-tomography (ET) was used to visualize the fine morphology of the isolated PSDs and calculate total protein occupancy within the PSD structure. Immunogold labeling was utilized to quantify protein composition and distribution of key signaling and scaffold molecules. Although the mean surface area did not significantly differ between PSD types, the PSD thickness, as measured from Cryo ET reconstructions, differed significantly between PSD types. Labeling densities for PSD-95 and αCaMKII were found to differ dramatically among the PSD types, while all regions had moderate to high labeling for βCaMKII, illustrating the importance of βCaMKII to the PSD structure. PSD-95, a scaffold protein, was absent from a fraction of cerebellar PSDs, unlike hippocampal and cortical PSDs, showing that protein composition varies between PSD types. Ripley's K function analysis of immunogold labeled PSDs showed that PSD-95 was clustered in cerebellar PSDs, unlike other PSD types, suggesting a different function for PSD-95 in cerebellar PSDs. In contrast, βCaMKII was found to have similar non-random organization in all PSD types. These results support the idea that the composition and structure of the PSD are modified to achieve the specific synaptic functions required of each brain region.